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PRINCIPAL INVESTIGATOR: Geoffrey M. Wahl, Ph.D.

CONTRACTING ORGANIZATION: The Salk Institute for Biological Studies
La Jolla, California 92037-1099

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We showed that extrachromosomally amplified genes in double minute chromosomes (DMs) are removed from tumor cells through preferential incorporation into micronuclei that are generated in S-phase. This process is enhanced by DNA synthesis inhibitors, and requires p53 inactivation. In the past year, we developed technology that enables visualization of chromosome dynamics in living cells. We made a fusion gene between human histone H2B and *Aequorea victoria* green fluorescent protein (H2B-GFP) to enable in vivo chromosome decoration. H2B-GFP was incorporated efficiently into nucleosomes, and chromatin that labeled brilliantly did not affect cell cycle progression. High resolution imaging of labeled live cells by time lapse confocal microscopy enabled observation and documentation of chromosome dynamics throughout the cell cycle. A retroviral vector was developed to apply this labeling strategy to a variety of cancer cell lines, including those harboring DMs. We could analyze for the first time the distribution of DMs in living cells during mitosis, and the formation of micronuclei. This strategy will facilitate the identification of DMs in tumor cells without cytogenetics, and more importantly, should provide a new and efficient approach to screen for chemotherapeutic agents that enhance DM elimination through micronucleation.

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Introduction

Gene amplification is manifested by autonomously replicating, acentric, extrachromosomal chromatin structures called double minute chromosomes (DMs) in as many as 50% of human tumors (1). DMs encode proteins involved in various facets of signal transduction and cell cycle controls. As DMs lack centromeres, they are maintained only when the overexpression of the gene products they encode enhance cell growth or survival. Importantly, DMs are never detected in normal cells, and they can be generated only in cells in which the p53 cell cycle control pathway has been inactivated (2). One reason that DMs may be so prevalent in cancer is that p53 pathway inactivation occurs in the vast majority of human malignancies including breast cancer (3). As DMs clearly constitute a genetic aberration restricted to neoplastic cells, and they can be rate limiting for growth (4, 5, 6), the long term goal of this research has been to develop strategies to efficiently eliminate DMs from tumor cells.

We previously showed that DMs are preferentially incorporated into micronuclei (4, 7). This was expected based on accepted models of mitotic micronucleation in which acentromeric DNA, like DMs, become enclosed in micronuclei, because such structures lag behind as chromosomes are drawn to opposite spindles in part by centromere-mediated motors. However, the progress report last year reported a novel mechanism of DM elimination involving de novo micronucleation during the cell cycle phase in which DNA replicates (S-phase). This novel mechanism led us to assess whether DNA synthesis inhibitors increase S-phase micronucleation. Indeed, we found that reducing nucleotide precursor pools via inhibition of ribonucleotide reductase (e.g., hydroxyurea, deferoxamine, guanazole, etc.), de novo uridine biosynthesis (using PALA), or chain elongation (using aphidicolin) greatly increased the efficiency of S-phase micronucleation. Interestingly, inactivation of the p53 tumor suppressor protein increased micronucleation frequency. Our data indicate that loss of p53 function increases the probability of forming the broken DNA fragments that initiate micronucleus formation during S-phase. We also reported that micronucleation was observed in tumors that contain DMs when they are growing in nude mice, suggesting that micronucleation is a common mechanism of DM elimination in cancer cells in vivo.

These observations raised important questions that we addressed during the past year. First, one report indicated that S-phase micronucleation might be a manifestation of apoptosis (8). We, therefore, determined the extent to which apoptosis contributes to S-phase micronucleation in the cells employed for our studies. Second, to understand the mechanism of S-phase micronucleation, we needed to develop a system to analyze the kinetics of micronucleus production in living cells. Our previous data suggested the existence of long and short lived micronuclei, and we needed to be able to observe the formation of each type and measure their respective lifetimes. The mechanism by which DNA synthesis inhibitors induce micronucleation also remains to be clarified. Most importantly, we have leads on new drugs that can increase S-phase micronucleation leading to DM capture and elimination, but a method to enable a high throughput screen was needed to expedite detection of additional agents that would be

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more useful in the clinic than those identified thus far. Answering the latter questions required a new technology to enable direct, real time observation of the micronucleation process in dividing cells. An approach to real time analysis of micronucleation was reported in the last progress report. We described the construction of a novel vector encoding the human histone H2B fused to the *Aequorea victoria* green fluorescent protein (H2B-GFP). This vector enabled fluorescent labeling of chromosomes in living cells. The data presented below documents some significant problems we encountered with the initial H2B-GFP gene cloned into this vector, and describes additional studies pursued over the past year which corrected and improved this system. We can now use the H2B-GFP approach to analyze a wide variety of cancer cells.

Methods and Results

I.   S phase micronucleation is different from apoptosis

The S-phase micronucleation process, described in the progress report last year, resembles the induction of "nuclear anomalies" by an apoptotic mechanism following γ-irradiation (8). However, the absence of highly condensed DNA in the nuclei producing budding micronuclei suggested that they were not undergoing apoptosis at the time of bud formation. In order to determine whether budding and micronucleation are separable from apoptosis, we determined whether micronuclei in COLO320DM cells contain the condensed, fragmented DNA that typifies apoptotic cells. Fragmented DNA was detected using the TUNEL assay (9). The results demonstrated that micronucleating cells did not stain by the TUNEL assay and did not exhibit the pycnotic structure of the apoptotic nucleus. A time course experiment demonstrated that budding increases during a single S-phase (rising to a maximum of approximately 5% of nuclei with buds) while the fraction of cells undergoing apoptosis remained roughly constant (0.5-1%). These observations lead us to propose that S phase micronucleation in COLO320DM occurs by a mechanism that does not require prior engagement of the apoptotic program.

II.   Observing chromosome dynamics in living cells

1)   Cloning of an H2B gene that does not interfere with cell cycle progression

The H2B-GFP expression vector described in the last progress report enabled vivid staining of chromosomes after transient transfection. However, stable cell lines expressing H2B-GFP were difficult to obtain. DNA sequence analysis revealed that the H2B clone used in the original vector contained two alterations that produced two amino acid substitutions in the histone fold domain. We reasoned that this could readily affect incorporation of H2B-GFP into nucleosomes, and might affect cell viability. These findings led us to analyze additional H2B clones, and to identify one lacking any mutations. One such clone was used to prepare a second set of H2B-GFP expression vectors. The new vector labeled chromosomes more robustly than the original vector. The following experiments employed this second H2B-GFP that encodes a normal H2B moiety.

2) H2B-GFP protein is incorporated into nucleosomes

We first generated a HeLa cell line that contains the H2B-GFP expression construct (H2B-G, Fig. 1) integrated stably in its genome. The cell line obtained expresses the construct from a constitutive high efficiency promoter (EF1α), and the cells in the population exhibit high level, uniform staining with H2B-GFP (see below). H2B-GFP was expressed stably in this cell line for the length of the analysis (>three months). This cell line has a high mitotic index and its growth rate is similar to that of the parental HeLa cell line. These data indicate that the robust H2B-GFP expression is compatible with prolonged cell growth, and does not produce detectable changes in growth characteristics.

We next determined whether chromosome staining is due to incorporation of H2B-GFP into nucleosomes, or to non-specific chromatin binding. Nucleosome core particles were fractionated biochemically and analyzed for the presence of H2B-GFP. Mononucleosomes were generated by extensive micrococcal nuclease digestion of the isolated nuclei expressing H2B-GFP. Fractionation of mononucleosomes demonstrated that H2B-GFP is incorporated into nucleosomes (Fig. 2). This experiment also shows that H2B-GFP comprises approximately one third of the endogenous H2B protein.

It was conceivable that GFP tagging of H2B protein could affect chromatin structure sufficiently to perturb cell cycle progression, especially during S-phase when histones of parental nucleosomes are re-distributed to newly replicated DNA. We therefore analyzed the cell cycle distribution of the established H2B-GFP cell line to investigate this possibility. The green emission of GFP labeled cells produces an approximately three-log shift from parental HeLa cells. DNA content was determined by measuring red emission of PI. The results indicate that the cell cycle distribution of asynchronous HeLa cells expressing H2B-GFP is indistinguishable from that of the parental HeLa cells (Fig. 3). Therefore, despite incorporation of H2B-GFP into a significant number of nucleosomes, any perturbations in chromosome architecture or function were not detectable at the limits of flow cytometry.

Living cells with H2B-GFP expression were observed by confocal microscopy to determine the pattern of chromatin staining in interphase and mitotic cells. H2B-GFP enabled highly sensitive chromatin detection in all phases of the cell cycle in living cells (Fig. 4). Fixation and permeabilization of the cells, which might cause artificial distortion of intracellular structure, is not required to obtain such images. H2B-GFP is highly specific for nuclear chromatin since no fluorescence was observed in the cytoplasm. We conclude that H2B-GFP allows highly sensitive, high resolution imaging of chromosomes in living cells in a fashion that does not measurably affect the dynamics of DNA replication or cell division.

3) Time-lapse observation of living chromosomes

One purpose of this experimental system is the analysis of chromosome dynamics in living cells, and eventually analysis of the micronucleation process in untreated and treated cells. To assess the

generality of the H2B-GFP labeling system, and to begin to use it to study chromosome dynamics, we needed a cell line with a high mitotic index that also contains DMs. We chose the Chinese hamster cell line 5P20 as it meets both criteria. We then used time-lapse confocal microscopy to observe chromosome dynamics in living 5P20 cells. Fig. 5 shows preliminary time-lapse analysis of a 5P20 cell undergoing mitosis using image collection every 30 seconds for 30 minutes. The experiment showed robust staining of all chromosomes, indicating that the human H2B can substitute for the H2B of other mammals with sufficient efficiency to enable uniform, bright chromosome decoration. No photobleaching occurred over this interval. The analysis reveals the progress of a tripolar mitosis in this unusual cell. Such aberrant figures are not unusual in p53-deficient cells as they often contain multiple centrosomes (10).

Long term analyses are required to study S-phase micronucleation in cells with DMs. We therefore performed a long duration experiment using an epifluorescence microscopy system in which one image was collected per minute over a period of 17hrs. This analysis used HeLa cells with H2B-GFP expression. Importantly, no photobleaching was observed, indicating the utility of this approach for long term analyses of chromatin dynamics in living cells.

4) Construction of retroviral vector and DM labeling

The H2B-GFP system has the potential to enable rapid visualization of nuclear DNA. However, its utility is compromised by the need to use transfection to introduce it into the cells to be analyzed. This can be a significant problem as many tumor cell lines exhibit low transfection efficiencies. This problem is emphasized by our inability to use transfection to establish a COLO320DM cell line exhibiting high, uniform H2B-GFP expression. Therefore, we constructed a retroviral vector to enable high efficiency transfer and expression of H2B-GFP. We employed a vesicular stomatitis virus G glycoprotein (VSVG) pseudotyped retroviral vector, as this system affords the highest viral titers available (11). High titer virus solutions were prepared 3 days after transfection into the packaging cell line. COLO320DM cells were infected with the H2B-GFP retrovirus, and 2 days later, nearly 100% of the cells expressed H2B-GFP protein. Fig. 6 shows an example of fixed COLO320DM cells infected with the H2B-GFP retrovirus. Double minute chromosomes were readily observed as small fluorescent dots with a sensitivity similar to that of FISH with a DM-specific probe (compare the DM intensity in the FISH analysis with that shown in the H2B-GFP image). We have routinely observed that the sensitivity of H2B-GFP staining exceeds that obtained with the DNA staining dye DAPI. Furthermore, DMs and micronuclei stained with H2B-GFP were, for the first time, observed in living COLO320DM cells. Experiments in progress are designed to study the dynamics of micronucleation during the life cycles of exponentially growing and drug treated COLO320DM and other cancer cells, including breast cancer cell lines.

III. DM specific labeling

In the last progress report, we raised the possibility that micronuclei containing DMs might enable gene transfer to occur between cancer cells, or possibly between cancer and normal cells. We began to investigate this possibility using extrachromosomally replicating EBV vectors encoding a drug resistance marker to model DM behavior. The EBV vector was transfected into COLO320DM cells. Unexpectedly, FISH experiments demonstrated that the transfected EBV vectors colocalized with DMs in drug-selected cells. We further analyzed this colocalization because, if covalent, such an association could enable specific targeting of transfected DNA to DMs. A procedure for tagging DMs with specific markers could prove useful for analyzing their ability to be transferred to target cells, as well as facilitate the analysis of their dynamics within the nucleus of a living cell as described below.

We examined whether the targeting efficiency depended on the presence of a viral replication origin in the vector, since we previously found that DM localization to the nuclear periphery correlated with DM replication. The EBV origin of replication and EBNA-1 gene were deleted from the original vector. After transfection into COLO320DM cells and drug selection, we established several clones containing this non-replicating transgene. The localization of the transgene was determined by FISH. The results indicated that non-replicating EBV vectors also associated with DMs; in this case, increased EBV copy number was observed in a certain percentage of the transfected cells. Since this vector cannot replicate autonomously in the transfected cells, it is reasonable to conclude that the copy number increase resulted from DM integration. However, we found that the replication-competent EBV vector was targeted to DMs more efficiently than the non-replicating vector. It remains to be determined whether this result reflects the increased copy number of the replicating vector, or to preferential colocalization of the DM and EBV vector at an intranuclear site (e.g., replication focus) prior to recombination. Of great interest, we were not able to detect recombination between transfected DNA and the DMs present in 5P20 cells. Interestingly, these DMs were not preferentially incorporated into micronuclei. We are now testing whether 5P20 DMs localize to the nuclear periphery. If they do not, this would raise the possibility that peripheral nuclear location may not be just a default location for acentric DNA, but may rather require the presence of sequences and/or structures within the DM that target them to this location. It is tempting to speculate that heterochromatic DNA, or proteins that bind heterochromatin, may be involved based on studies of the behavior of the X-chromosome Barr body (12) and heterochromatin binding proteins in Tetrahymena (13).

Conclusion and Experimental Prospectus

The studies conducted in the past year have significantly advanced our understanding of the mechanisms of micronucleation, the dynamic behavior of acentric fragments in mammalian cells, and they have provided a significant technical advance to facilitate additional studies in the coming year. The data summarized above rigorously demonstrate that H2B-GFP system offers unique advantages for

visualizing DMs in living cells in a way that does not perturb the cell cycle. This should enable us to study micronucleation using experimental designs that no longer require the synchronizing agents that may indirectly affect this process.

The retroviral vector should greatly broaden the utility of the H2B-GFP approach. In the next year, we will utilize it to detect DMs in primary cells obtained from tumors. This is an essential step for identifying patients who could benefit from chemotherapeutic protocols designed to reduce or eliminate DMs. Cytogenetic analyses are often impeded by the difficulty of preparing adequate metaphase spreads from tumor cells with low mitotic indices, such as those we encountered with breast tumor specimens. Such preparations may also contain debris and/or nuclear matrices that can obscure small chromatin elements such as DMs, and G-banding methods for chromosome visualization can actually erase evidence of lightly staining DMs. Since H2B-GFP staining is highly specific for chromosomes, it readily enables DM visualization even in living cells. Therefore, it should provide a rapid, sensitive method for identifying DMs in primary biopsy specimens. We have designed proof of principle experiments to detect DMs in dividing cancer cell lines within 24-48 hrs after infection of the H2B-GFP retrovirus. If the cell line work demonstrates feasibility of the approach, we will collaborate with clinical investigators who have protocols involving isolation of breast biopsy material to determine whether short term culture after retroviral infection provides evidence of DMs in such samples. Based on our previous experience with cytogenetic analyses of biopsy samples (B. Parker, G. Wahl, A. Melloni, A. Sandberg, unpublished data), we expect that approximately 30% of pleural effusions would contain DMs.

We continue to try to identify tumor cell lines with DMs. As stated previously, extended passage in culture often results in the outgrowth of variants which have lost the DMs, or in which the DM sequences are integrated into chromosomes. We are therefore contacting collaborators who are making mouse models involving orthotopic transfer of human breast cancer cells obtained at biopsy. If the H2B-GFP approach can be used to rapidly identify patient samples with DMs, we will attempt to establish mouse models using these cells for orthotopic transfer. We have initiated discussions with Dr. Dennis Slamon, a noted breast cancer specialist at U.C.L.A., as he has informed me that he has one breast cancer orthotopic model under analysis, although he has not determined whether the cells have DMs. A primary goal will be to determine if these cells do contain DMs, and if so, to use the micronucleus purification strategy we developed to determine the chromosomal location and possible candidate genes they encode.

The covalent association between the transgenes and DMs demonstrated that we could label DMs with a genetic marker in vivo. We propose to construct a targeting vector containing multiple copies of the lac repressor. Expression of GFP-tagged lac repressor in the same cells should enable the selective fluorescent decoration of DMs, as described for chromosomes by the elegant work of Belmont and colleagues (14). We have obtained this system from Dr. Belmont and are preparing the required

targeting vector. The availability of this system will enable us to study the dynamics of DM behavior within the nuclei of living cells, and will reveal details of the micronucleation process with a precision not achievable by any other method. Our prior experience with H2B-GFP system will greatly facilitate and expedite successful completion of this project.

We have learned much about the micronucleation process, but an important challenge that lies ahead is to identify clinically useful drugs useful for DM elimination through micronucleation. An important goal will be to use either the H2B-GFP approach, or the specific DM labeling method, to design a high throughput fluorescence based screen for drugs that enhance micronucleation and DM elimination. This is clearly an ambitious goal that we will probably just begin to develop during the coming year. Completion of this goal, and identification of new lead compounds, or elucidation of existing approved agents that enhance micronucleation, may both have mechanistic significance for understanding micronucleation, as well as avail patients of new chemotherapeutic strategies dictated by deeper understanding of the genetic changes that underlie tumor cell growth and survival.

Figure Legends

Figure 1

Schematic representation of H2B-GFP chimeric peptides

H2B peptide (239 a.a.) was tagged with GFP peptide (126 a.a.) either at its C-terminus (H2B-G) or its N-terminus (G-H2B). The number of extra amino acids at the junction of H2B and GFP peptides are indicated. Histone N-terminal tails are indicated as shaded boxes.

Figure 2

H2B-GFP is incorporated into mononucleosomes

(a)   Sucrose gradient analysis of mononucleosome populations. The mononucleosome protein-DNA complexes, prepared by micrococcal nuclease digestion, were purified through parallel 5-30% sucrose gradients. Proteins from each fraction were extracted and analyzed by 15% SDS-PAGE and Coomassie staining. H2B-GFP protein (approx. 45 kDa, shown by the arrows) and native core histone proteins are indicated.

(b)   The same aliquots of the protein samples were electrophoresed and analyzed by Western blotting using anti human H2B antibody. H2B-GFP protein and native H2B protein are indicated.

(c)   DNA was extracted from each corresponding fraction (a, b) and analyzed by 1.5% agarose gel.

Figure 3

H2B-GFP expression does not affect cell cycle progression

Non-labeled and H2B-GFP expressing HeLa cells were harvested by trypsinization, fixed in 70 % ethanol, and stained with 20 µg/ml propidium iodide (PI) containing RNase. Fluorescence of cells was

measured using a Becton Dickinson FACScan. The red (PI) and green (GFP) emissions from each cell were separated and measured using standard optics of the FACScan.

(a)   Green emission of HeLa cells and H2B-GFP expressing cells.

(b)   DNA histogram of the same cells as shown in (a) determined by PI staining.

Figure 4

Localization of H2B-GFP protein

Confocal microscopic images of various stages of the live HeLa cells expressing H2B-GFP. Interphase (a), prophase (b), metaphase (c), and anaphase (d) cells are shown. In each panel, the GFP signal is shown in green at left and with a differential interference contrast (DIC) image on the right.

Figure 5

Time-lapse imaging of H2B-GFP labeled 5P20 cell undergoing anaphase-telophase transition in tripolar mitosis. Images were collected at 30 second intervals using time-lapse confocal microscopy.

Figure 6

Double minute chromosomes (DMs) and micronuclei are visualized using H2B-GFP

(left)   Biotinylated c-myc cosmid probe was hybridized to metaphase and interphase COLO320DM cells by FISH. Signals were detected with avidin-FITC. Slides were counterstained with propidium iodide (2 µg/ml) and observed by fluorescence microscopy utilizing a triple band filter.

(right) H2B-GFP localization. COLO320DM cells expressing H2B-GFP were treated with colcemid (100 µg/ml for 1 hr), swollen in a hypotonic solution, cytospun, and fixed with 3.7% formaldehyde. Slides were observed by fluorescent microscopy utilizing a FITC filter.

(top)   DMs harboring amplified c-myc genes are designated by the arrows.

(bottom)   Micronuclei incorporating DMs are shown by the arrows.

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Fig 1

Fig 2

a

KDa
104
80
47
34
28
20

HeLa   H2B-GFP

H2B-GFP →

H3
H2B
H2A
H4

b

KDa
104
80
47
34
28
20

H2B-GFP →

H2B →

c

200 bp
100 bp

1 2 3 4 5 6 7 8 9 10 11 12

16
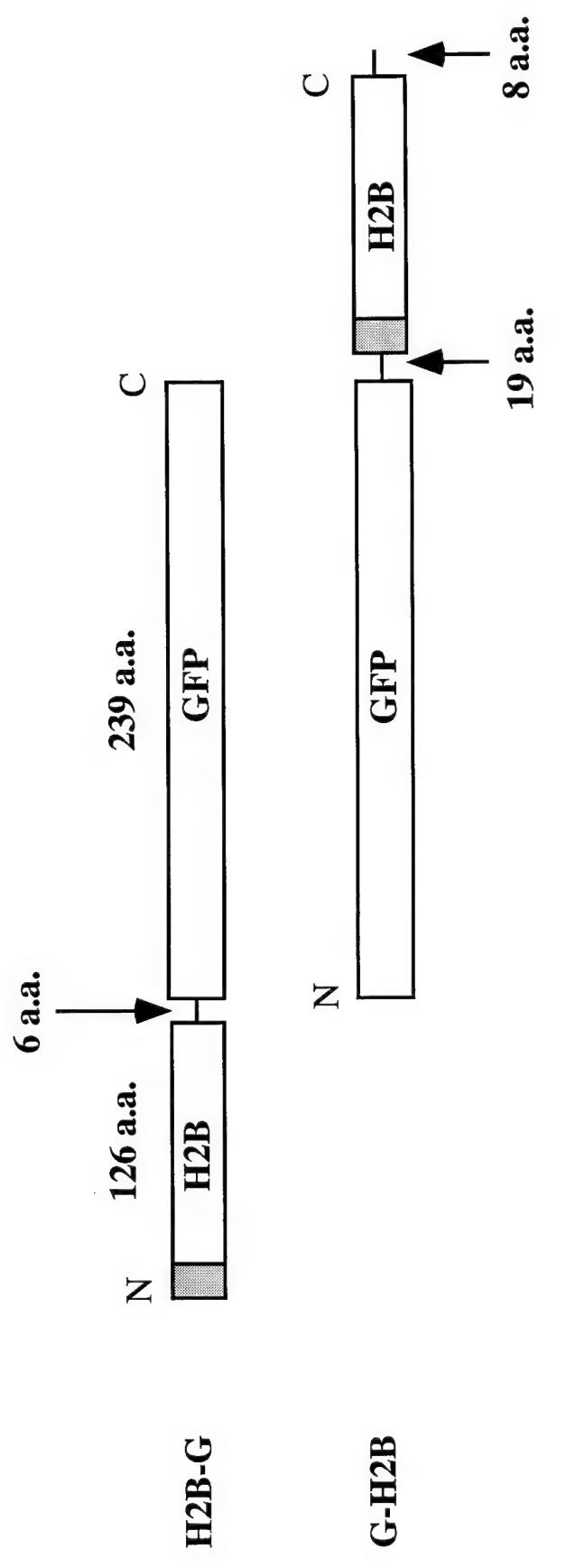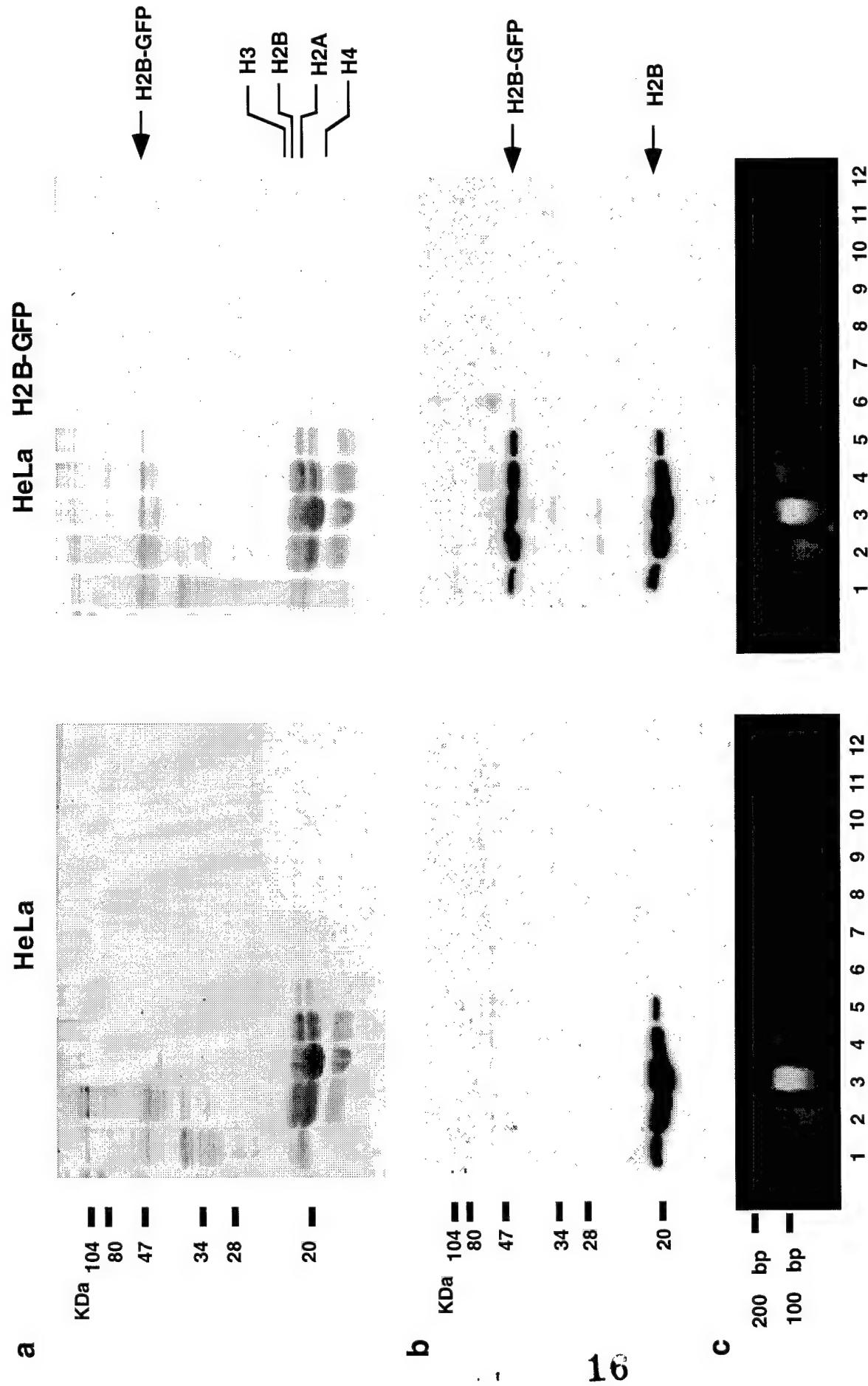
Fig 3

HeLa **HeLa with H2B-GFP**

a

Cell Number

Green Fluorescence

Green Fluorescence

b

Cell Number

G1:   37.7%
S: 35.4%
G2/M: 26.9%

G1:   34.2%
S: 36.1%
G2/M: 29.7%

DNA content (PI)

DNA content (PI)
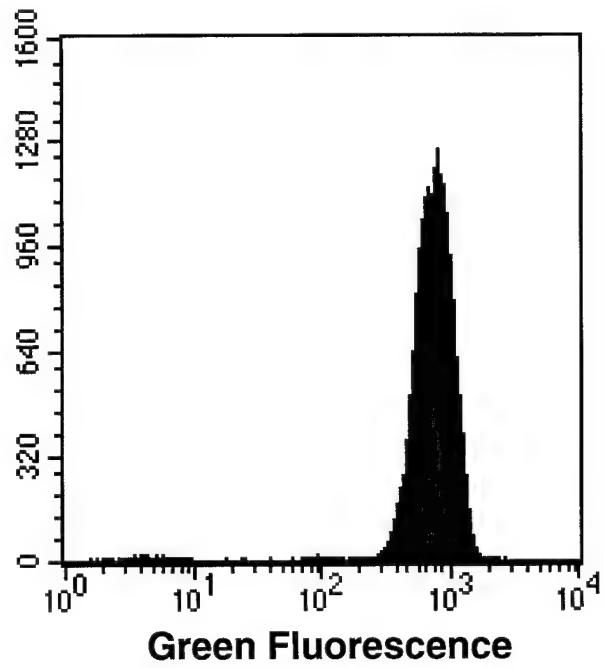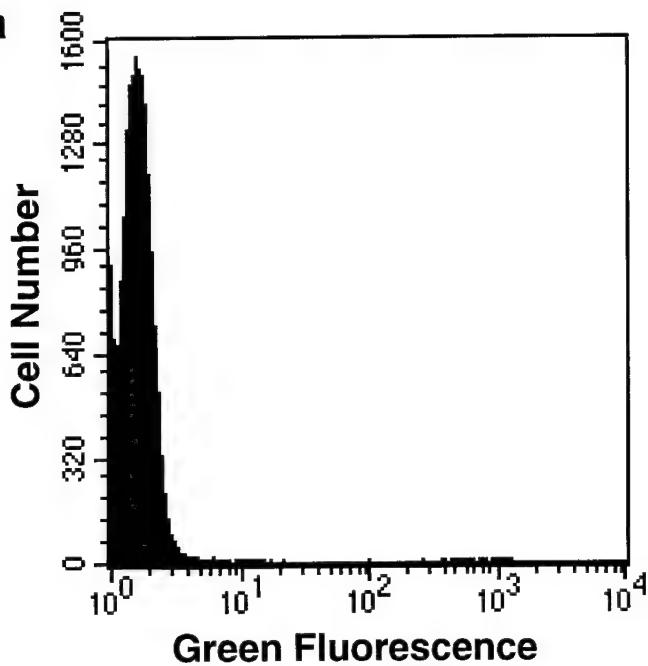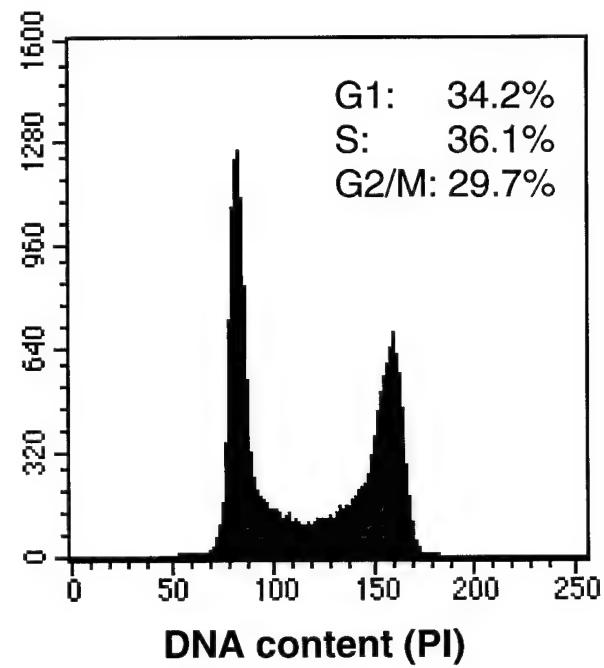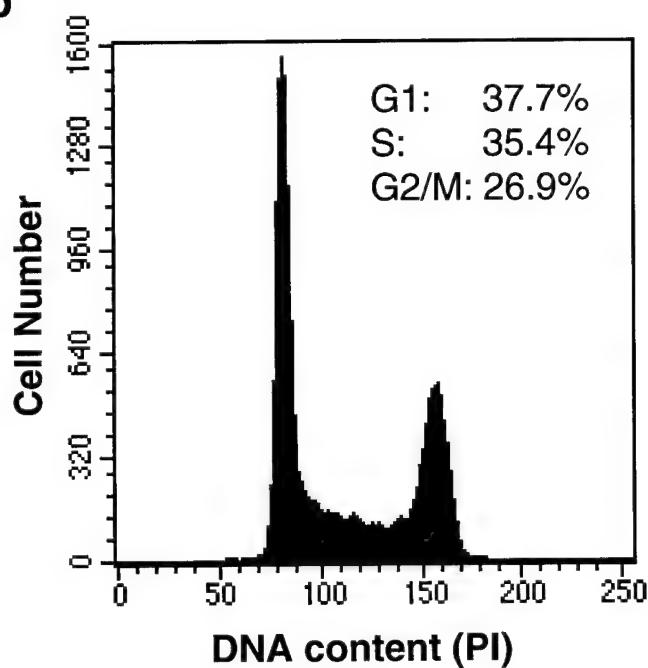
Fig 4

a
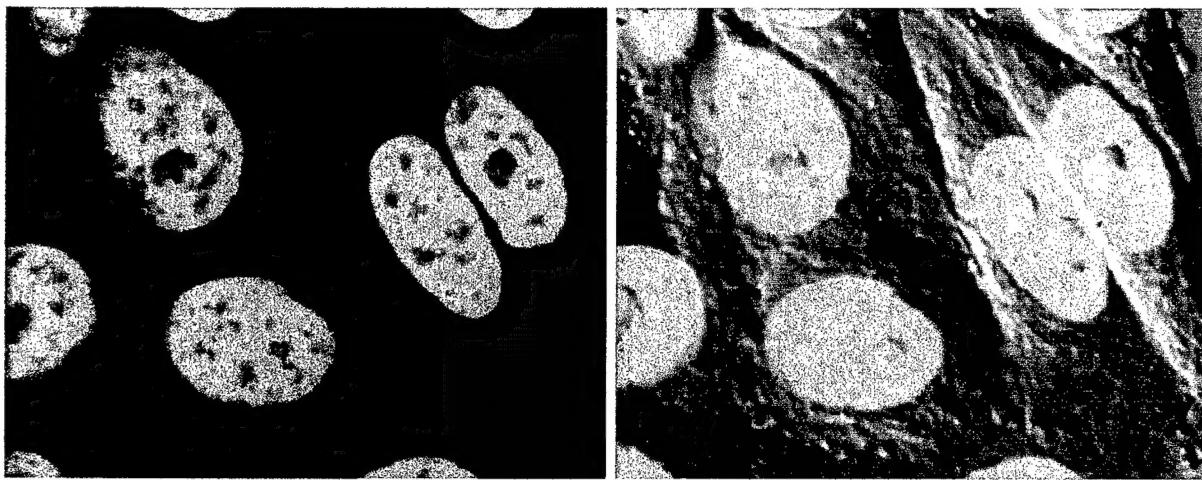
b
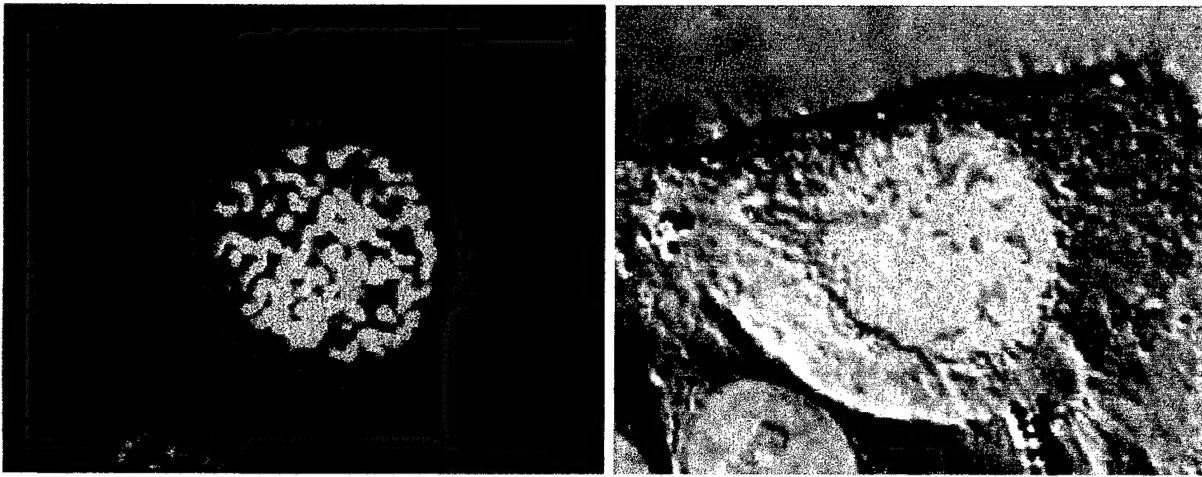
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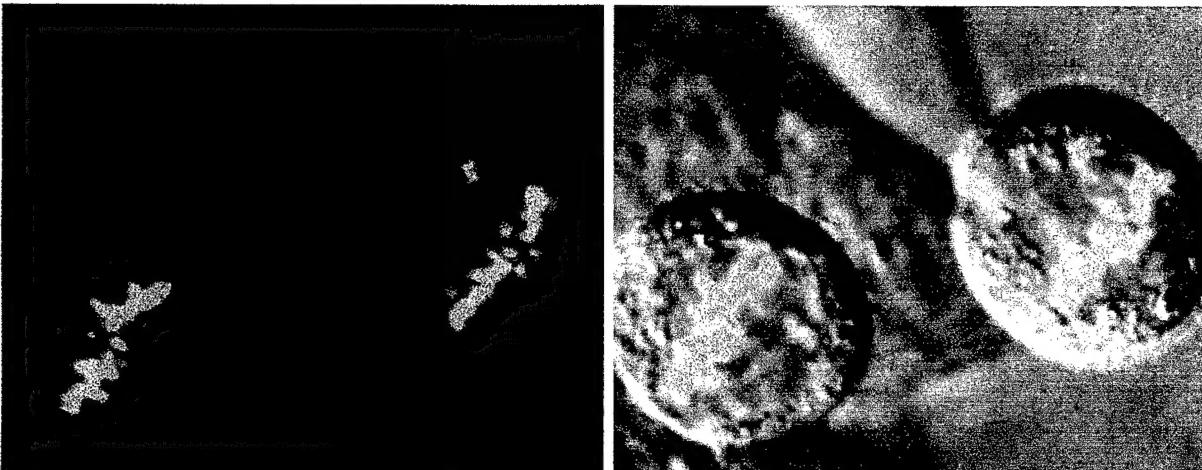
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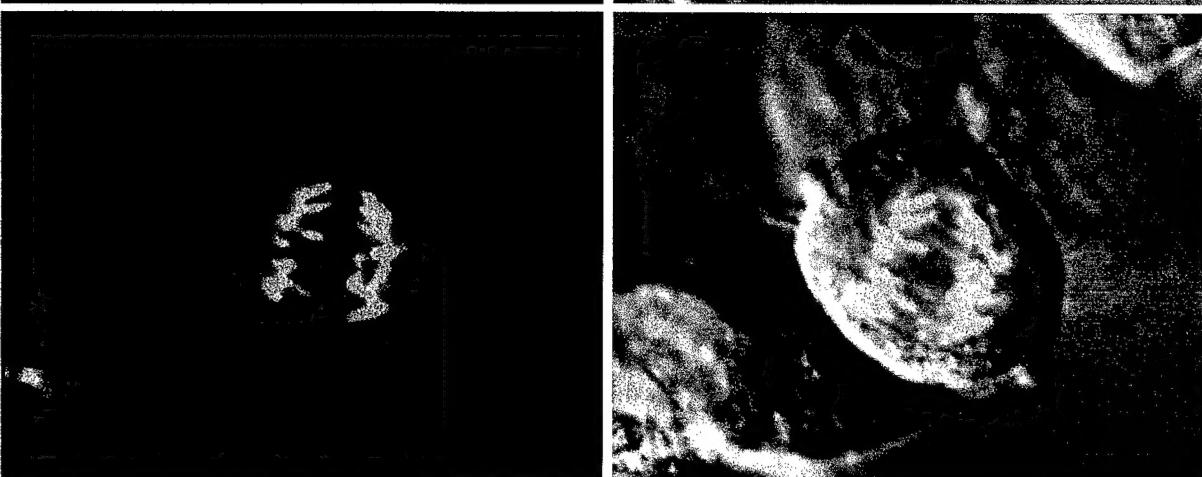
Fig 5

20 min

0 min

30 min

10 min

Fig 6

H2B-GFP localization

FISH
with c-myc cosmid probe

COLO320DM
metaphase

COLO320DM
interphase
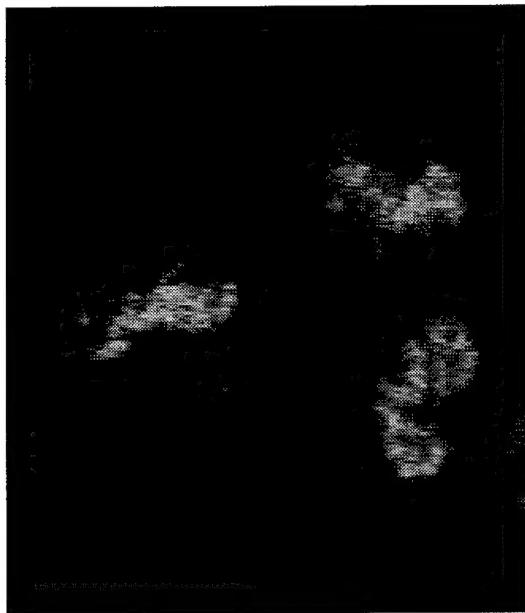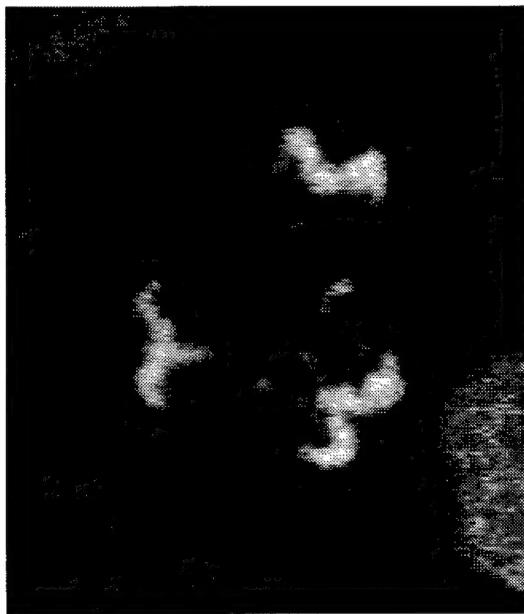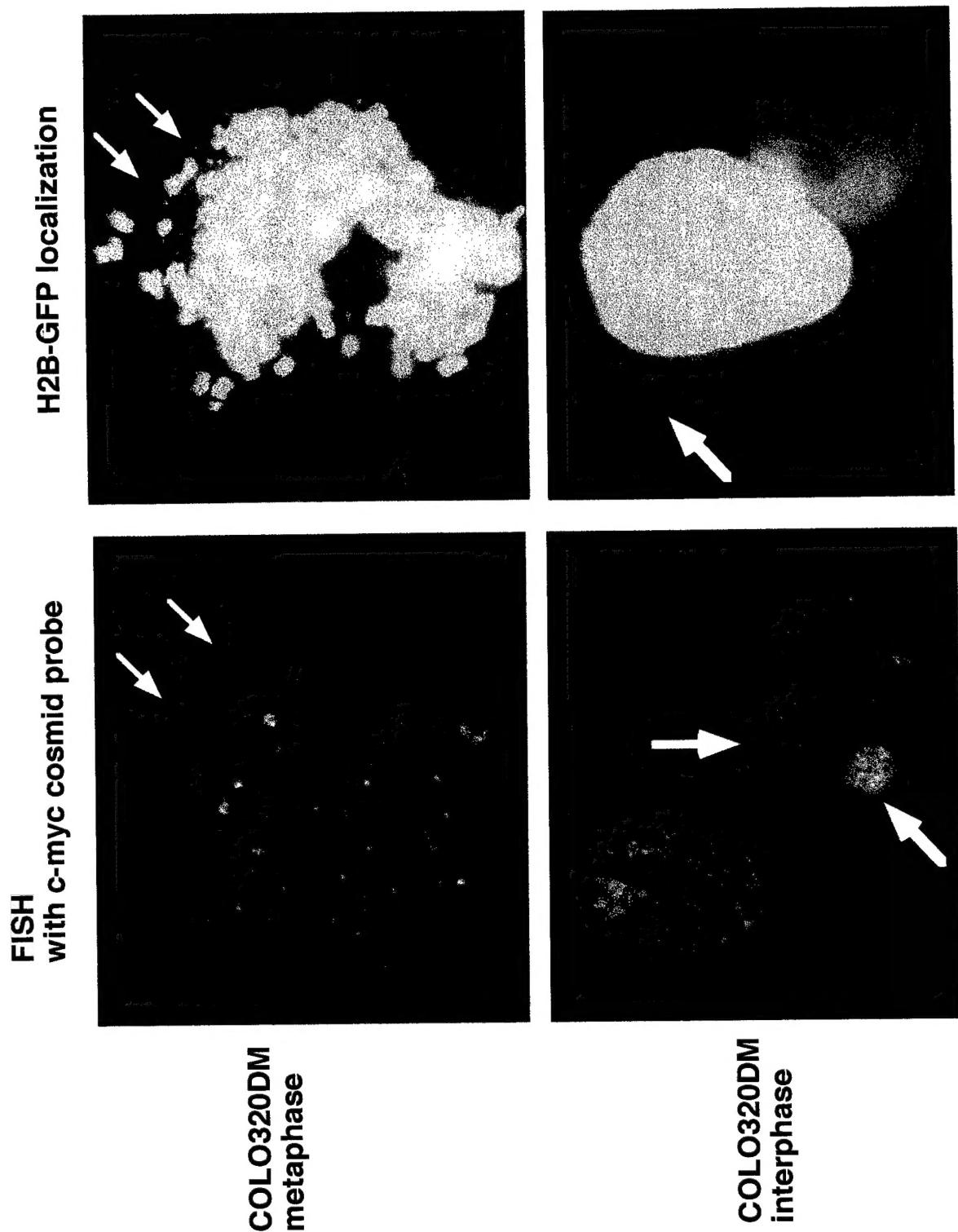
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Kanda T., and Wahl G.M. (1997) A histone GFP fusion protein useful for analyzing chromosome segregation in living cells. (submitted).

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1. Geoffrey M. Wahl, Ph.D. Principal Investigator
2. Teru Kanda, M.D., Ph.D., Post-doctoral Fellow
3. Omid Vafa, Ph.D., Posst-doctoral Fellow (began 7/97)
4. Kristie C. Clarkin, Research Associate (9/96 - 12/96)
5. Maoxiim Urista, Undergraduate Research Assistant (9/96 - 11/96)